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The occurrence of honeybee viruses in apiaries in the Koprivnica-Križevci district in Croatia

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ABSTRACT

Viral honey bee diseases are an increasing beekeeping problem throughout the world because they can cause great economic losses and a reduction in biodiversity in natural ecosystems. Therefore, it is important to promptly identify the causal pathogens of honey bee diseases in order to develop appropriate measures and procedures for their prevention and eradication. In this paper the prevalence and distribution were determined of the five economically most important honey bee viruses in the Koprivnica-Križevci district by Reverse-transcriptase PCR (RT-PCR). In addition, the honey bee samples were examined for the presence of the mite *Varroa destructor*. In the investigated apiaries the following viruses were identified: Deformed wing virus (DWV) with 100%, Sacbrood bee virus (SBV) with 70%, Black queen virus (BQCV) with 20%, Acute bee paralysis virus (ABPV) with 10% and Chronic bee paralysis virus (CBPV) with 10% incidence. Multiple infections of the examined honey bee colonies were found in 80% of samples.

Key words: honey bee viruses, reverse-transcriptase PCR, Koprivnica-Križevci district

Introduction

Among honey bee pathogens, viruses are one of the most important threats to the health and well-being of bees and can cause serious concern. The majority of honey bee virus infections are unidentifiable by beekeepers because they are usually unapparent and/or persistent (HUANG et al., 1996). Also, in combination with varroa infestation, virus infections have been shown to reduce the lifespan of bees (HIGHFIELD et al., 2009; MATAŠIN et al., 2012) and are frequently implicated in collapsing colonies (CARRECK et al., 2010; MARTIN et al., 2012). However, a variety of other weakening factors such as

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nosema disease, intoxications or environmental circumstances may play a role in the clinical manifestation of virus infections (ALLEN and BALL, 1996).

The most of important honeybee viruses are single stranded RNA viruses and include Acute bee paralysis virus (ABPV) and Black queen cell virus (BQCV), which are classified as members of the genus *Cripavirus* of the family *Dicistroviridae*; then Deformed wing virus (DWV), Sacbrood virus (SBV) as members of the genus *Iflavirus*; and Chronic bee paralysis virus (CBPV) which remains unclassified. ABPV is an infective agent of bees with worldwide distribution (ELLIS and MUNN, 2005) commonly existing as covert low-titer infections, but severe infection can produce observable symptoms, such as paralysis-trembling-inability to fly (RIBIERE et al., 2008), or a sharp decline in the adult population (TODD et al., 2004). BQCV affects the honeybee brood, and typical signs are enlarged dark stained queen cell walls (SIEDE and BUCHLER, 2003). CBPV causes a contagious disease of adult honey bees characterised by trembling, sightlessness and hairless-black individual crawling at the hive entrance (RIBIERE et al., 2010). SBV primarily affects the honey bee brood, infected larvae fail to pupate and ecdysial fluid aggregates around the integument and finally results in larval death (RITTER, 1996). DWV is the most common and widely distributed honey bee virus and causes symptomatic crippled-wing syndrome, often seen in bees heavily infected with varroa (MÖCKEL et al., 2011; DAINAT et al., 2012).

The Koprivnica-Križevci district is a significant geographic and traffic location in relation to other parts of Croatia but also because of its vicinity to other EU member states, as well as to several non-EU states, which makes it important to investigate the status and the regional distribution of honeybee pathogens. There is no literature pertaining to the prevalence of honeybee viruses in the examined area, probably because of the lack of information on the specific clinical signs, and unavailable diagnostic tools, and consequently the lack of objective data about viral disease outbreaks. Although laboratory molecular diagnosis supported the presence of ABPV, CBPV, DWV, SBV and BQCV, no samples tested positive for Kashmir bee virus (KBV) or Israeli acute bee virus (IAPV), in all twenty districts and all three climatic areas (Mediterranean, mountain and continental) of Croatia in 2010 (TLAK GAJGER et al., 2014).

The aim of this survey was to estimate the occurrence of the five most economically important honey bee viruses in apiaries situated in the Koprivnica-Križevci district in Croatia, and to obtain a comprehensive insight into the correlation between the presence of honey bee viruses, their clinical manifestation and the presence of the mite *V. destructor*.

Materials and methods

Beekeepers from ten different locations (microlocations) situated in the Koprivnica-Križevci district (Fig. 1) submitted honey bee samples from colonies suffering from symptoms of depopulation and/or varroa infestation. Samples were collected from the

inside of beehives, directly from combs during the inspection of honeybee colonies, during the active beekeeping season in 2011. Each sample consisted of approximately 300 bees, representing one location or apiary. The samples were stored at -20 °C prior to examination. Thirty to fifty adult bees were randomly selected from each sample and subjected to viral RNA isolation and subsequent Reverse-transcriptase PCR (RT-PCR) analysis. The rest of the bees from each sample were used for parasitological examinations for *V. destructor* mites by washing the bees with warm soapy water.

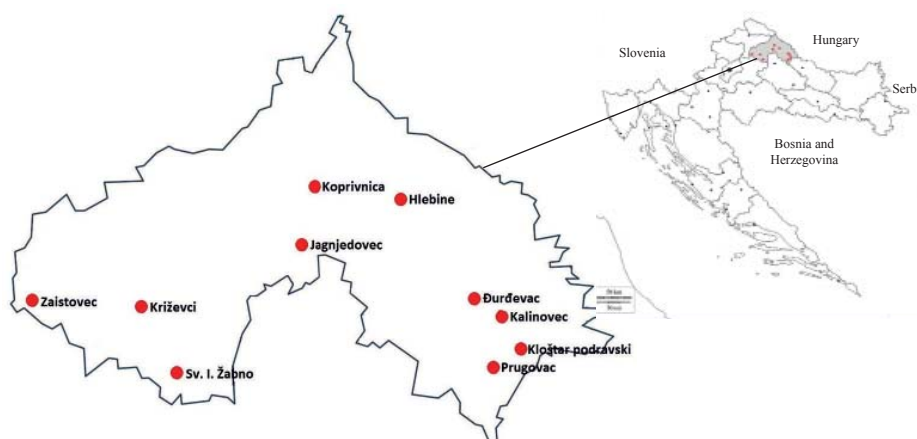


Fig. 1. Honey bee sampling microlocations at Koprivnica-Križevci district in Croatia

Part of each sample separated for virological examination was homogenized in sterile plastic containers with sterile sand, and suspended in 10 mL diethylpyrocarbonate treated water. The mixture was centrifuged for 15 minutes at 11000 G and 200 µL was used for viral RNA extraction. Viral RNA was extracted employing the QIAmp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Primers used for honeybee virus detection in RT-PCR assays were according to BERENYI et al. (2006). Amplification was performed in a 0.25 µL 1.25 U Taq DNA polymerase, 3 µL (NH₄)₂SO₄, 10XTaq buffer, 3 µL 3 Mm MgCl₂, 0.5 µL 10 Mm dNTP mix, 0.5 µL 0.4 µM primer, 0.5 µL 0.4 µM reverse primer, 19 µL distilled water and 3µL cDNA (Promega, USA). PCR amplifications were performed in a Prime PCR system (Bibby Scientific Ltd, UK). The reverse transcription at 50 °C for 40 min was followed by denaturation and polymerase activation at 95 °C for 15 min, 40 cycles at 95 °C for 30 sec, at 60 °C for 1 min and at 66 °C for 2 min, and a final elongation step for 7 min at 72 °C. Reaction mixtures without RNA served as negative controls and previously verified positive samples as positive

controls. An aliquot of 10 µL of the reaction product was electrophoresed in the Lonza FlashGel system, visualized and photographed by Lonza FlashGel Camera and Capture Software 3.1 (Lonza Rockland. Inc., USA). A 100bp ladder (Fermentas, USA) was used as the standard for fragment size determination.

Results

Ten pooled samples of adult honey bees originating from the affected honey bee colonies, from 10 different apiaries, situated in the Koprivnica-Križevci district, were collected and tested for the presence of five honey bee viruses by specific RT-PCR methods. The results showed that 100% of investigated samples were infected with DWV, 70% with SBV, 20% with BQCV, 10% with ABPV and 10% with CBPV. Multiple infections of examined honey bee colonies were found in 80% of samples. Laboratory examinations revealed that 80% of honey bee samples were positive for varroa presence. All results are presented in Table 1 and Figs 2 to 6. Regional differences in the distribution of five examined viruses were not ascertained.

Table 1. Examination results of honey bee samples originating from the Koprivnica-Križevci district for viruses and *V. destructor* presence

No.	Sampling location and code number	SBV	BQCV	DWV	ABPV	CBPV	<i>V. destructor</i>
1	Prugovac, 48362	pos.	neg.	pos.	neg.	neg.	pos.
2	Koprivnica, 48000	neg.	neg.	pos.	neg.	neg.	neg.
3	Đurđevac, 48350	neg.	neg.	pos.	neg.	neg.	neg.
4	Hlebine, 48327	neg.	pos.	pos.	neg.	neg.	pos.
5	Jagnjedovec, 48000	pos.	neg.	pos.	neg.	pos.	pos.
6	Sv. I. Žabno, 48214	neg.	neg.	pos.	neg.	neg.	pos.
7	Kalinovec, 48361	pos.	pos.	pos.	neg.	neg.	pos.
8	Križevci, 48260	pos.	neg.	pos.	neg.	neg.	pos.
9	Kloštar Podravski, 48362	pos.	neg.	pos.	neg.	neg.	pos.
10	Zaistovec, 48267	pos.	neg.	pos.	pos.	neg.	pos.

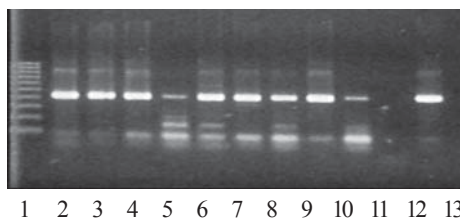


Fig. 2. RT-PCR results of honey bee samples for deformed wing virus (434 pb)

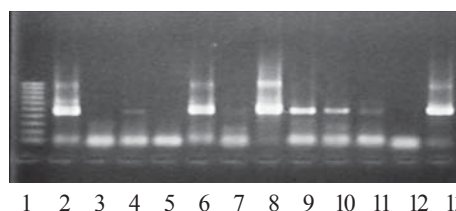


Fig. 3. RT-PCR results of honey bee samples for Sacbrood virus (487 pb)

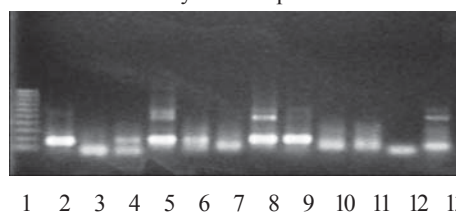


Fig. 4. RT-PCR results of honey bee samples for Black queen cell virus (472 pb)

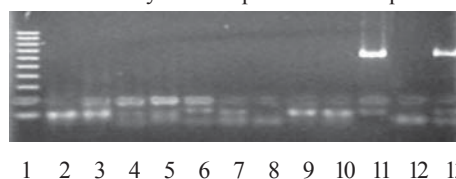


Fig. 5. RT-PCR results of honey bee samples for Acute bee paralysis virus (618 pb)

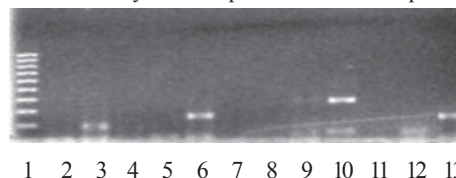


Fig. 6. RT-PCR results of honey bee samples for Chronic bee paralysis virus (315 pb)
Column 1 - standard marker (100 pb); column 2 to 11 - PCR products of examined samples; column 3 - negative controls; column 4 - positive controls

Discussion

In this research article we describe the molecular-genetic evidence of five viruses in samples of honey bees collected from apiaries in the Koprivnica-Križevci district, as a base for establishing epidemiological surveillance of honey bee diseases in the continental part of Croatia. The main reason for this is the fact that diagnosis of virus infections is the key component of honey bee diseases for surveillance, monitoring and control

programs (CHEN et al., 2006). The significance of viral diseases is still not completely appreciated because they are widespread and often cause unapparent, multiple infections in seemingly healthy honey bee colonies. In Croatia, until recently, viral bee diseases were not systematically investigated, and this is the first study to link viruses with varroa infestations.

Of the five viruses identified by RT-PCR, DWV had the highest prevalence (100%), which supports the published data that DWV can persist in honey bee colonies as an unapparent infection, and the high prevalence is similar to the prevalence reported previously (TENTCHEVA et al., 2004; BERENYI et al., 2006; NIELSEN et al., 2008; OKUR GUMUSOVA et al., 2010). With 70% prevalence, SBV was the second most prevalent virus. When we compare this high prevalence with SBV occurrence in other parts of Croatia, it is almost double (TLAK GAJGER et al., 2014), but for all positive cases in the examined areas beekeepers reported clinical manifestation of sac brood disease. Much higher (100%) SBV prevalence was reported in Uruguay (ANTUNEZ et al., 2005), and 86% in France (TENTCHEVA et al., 2004), and lower (48%) in Austria (BERENYI et al., 2006) and Slovenia (8.3%) (TOPLAK et al., 2012). BQCV was detected in 20% of examined samples of adult bees, which is a lower percentage in comparison with other parts of Croatia (30%) or other members of the EU. Surprisingly, the occurrence of ABPV and CBPV was 10% and mortality or clinical symptoms of paralysis- flightless- trembling or crawling bees were often evident in apiaries with these infections. In some EU countries, ABPV has been detected, with a high prevalence of 40% in Slovenia (TOPLAK et al., 2012), in France 58% (TENTCHEVA et al., 2004), in Hungary 67% (BAKONYI et al., 2002), and in Austria 68% (BERENYI et al., 2006). 80% of examined honey bee samples had more than one virus present at the same time, suggesting that many bee viruses are widely distributed in apiaries in this part of Croatia.

In this study, *V. destructor* was detected in 80% of samples that were also infected with multiple infections of honey bee viruses. Similar results were reported by BERENYI et al. (2006), and were expected because *V. destructor* is probably the most significant or predisposing factor for virus diseases and, with increasing resistance to some acaricides, could potentially be a high risk for colony losses.

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SAŽETAK

Virusne bolesti medonosne pčele predstavljaju ozbiljan problem u pčelarstvu diljem svijeta, jer uzrokuju velike gospodarske štete i smanjenje bioraznolikosti prirodnih ekosustava. Stoga je bitno pravovremeno utvrditi uzročnike bolesti kako bi se mogle poduzeti odgovarajuće mjere i postupci za sprečavanje pojave i njihovo suzbijanje. U ovom je radu lančanom reakcijom polimerazom uz prethodnu reverznu transkripciju utvrđena prisutnost i rasprostranjenost pet najznačajnijih pčelinjih virusa na području Koprivničko-križevačke županije. Dodatno su uzorci pčela pretraženi na prisutnost grinje *Varroa destructor*. Na pretraženim uzorcima pčela utvrđena je RNA virusa izobličanih krila u 100%, virusa mješastog legla u 70%, virusa crnih matišnjaka u 20%, virusa akutne pčelinje paralize u 10% i virusa kronične pčelinje paralize u 10% uzoraka. Višestruke mješovite infekcije u pretraženim pčelinjim zajednicama utvrđene su u 80% uzoraka.

Ključne riječi: pčelinji virusi, reverzna transkriptaza-PCR, Koprivničko-križevačka županija
